PROTOCOL

Introduction

This document details the material and procedures for evaluating the efficacy of an antimicrobial textile. It is based on the American Association of Textile Chemists and Colorists AATTC 100 test method, with modification made to the method to include alternative test microorganisms, laundering and simulation of environmental stresses.

Purpose

The purpose of this study is to document the efficacy of the test substance against the test system (microorganisms) under the test parameters specified in this protocol.

Justification for the selection of Test System (microorganisms)

USEPA requires specific antimicrobial claims, made for articles containing antimicrobial pesticides sold in the United States, to be supported by relevant test system (microorganisms) and validated or Agency-evaluated test methods. The test microorganisms were selected by the sponsors to represent a broad spectrum of clinically relevant microbial physiologies.

Materials

Reagents, Media and Supplies

- Sufficient quantity of test fabric.
- Pure culture of test (microorganisms).
- Sufficient quantity of 25 x 150mm test tubes containing 10ml sterile AOAC nutrient broth or AOAC synthetic broth.
- Sterile 50ml centrifuge tubes containing 20 ml sterile AOAC- modified Letheen broth containing 5-15 sterile glass beads.
- Sufficient sterile tubes containing 0.9 ml sterile Phosphate-Buffered Saline (PBS).
- Sufficient volume of sterile trypticase soy agar (or other media as appropriate) for enumeration and plating.
- Forceps.
- Vortex mixer.
- Inoculation loops.
- Bunsen burner, microbiological incinerator, or micro-torch.
- Micropipettes and a sufficient quantity of appropriately sized sterile micropipette tips.
- Automatic pipettor (PipetAid or similar) and various sizes of sterile surgical pipets.
- Appropriate volume of 95% ethanol.
- Incubators capable of sustaining a temperature of 36 ± 1℃ .
- Certified satellite clock.
- Calibrated digital timer.
- Calibrated thermometers.
- Sufficient volume of Triton X-100.
- Sufficient volume of fetal bovine serum.
- Laundry washing machine.
- Laundry drying machine.

- Name brand (non-antimicrobial) laundry detergent (Tergitol NP 40) for use in laundry washing machine.
- Laminar flow hood.
- Sufficient quantity of sterile Petri dishes.

Procedure for Initial Bacterial Reduction (Non-Continuous Claim)

Preparation of Test Tubes containing Neutralization Broth

- Before the test begins, the neutralization tubes are prepared by adding 0.9 ml volumes to appropriately sized sterilized tubes.

Laundering, Environmental Stressing and Re-inoculation of Treated and Control Textiles

- A sufficient numbers of treated and control textile coupons are washed in a laundry washing machine at 85± 15℃ using brand name non-antimicrobial, non-ionic and non-chlorine containing laundry detergent followed by a standard rinse cycle and dried at 62-96℃ for a period of 20-30 minutes.
- Laundered samples are placed in a 36± 2℃ incubator with a relative humidity of 85-100% for 2 hours (± 10 minutes) followed by exposure to UV by placing in a class ‖ biological safety hood for 15± 2 minutes at 20-25 ℃ with UV light on (treated and control fabric are laid flat to fully exposed the fabric).
- After UV exposure, each carrier (treated and control) is inoculated with 0.100ml of re-inoculation culture to yield ≥ 1 x 10^4 CFU/ Carrier, and allowed to sit undisturbed for 15 ± 5 minutes at room temperature, at which time the next laundering cycle is initiated.
- *See preparation of Re-inoculation Culture Inoculum* for details of re-inoculation culture preparation.
- The 25th cycle will not contain laundry detergent for the purpose of removing residual detergent from previous cycles and in preparation for efficacy testing, but will receive the heat, UV and re-inoculation mentioned above.

Preparation of Test and Control Carriers

- Treated textiles are provided to the testing laboratory by the Sponsor as 2'' x 4'' coupons edge sewn to prevent unraveling during laundering and are treated in a manner proposed for manufacturing.
- Untreated control textile is provided to the testing laboratory by the Sponsor as 2'' x 4'' coupons edge sewn to prevent unraveling during laundering and representative of the treated textile without antimicrobial treatment.
- A carrier for the study is defined as a single layer of a 2'' x 4'' (treated or control) textile fabric.
- Control fabrics may be steam sterilized on a fast exhaust cycle for 20± 5 minutes to eliminate contaminant microorganisms after laundering but prior to testing.

- Treated fabrics will not be sterilized after laundering or before efficacy testing but will undergo sterility control confirmation detailed in *sterility Control (Testing and Control Carriers)* along with control carriers prior to or in parallel with efficacy testing.
- Test and control carriers are individually placed in sterile Petri dishes for testing.

Test and Microorganism Growth and Incubation Requirements

- *S. aureus,* and *P. aeruginosa* test and re-inoculation cultures are cultured in AOAC Nutrient Broth at 36± 1℃ for 24± 2 hours (daily culture) or 48± 2hours (test culture). All enumeration and controls are plated using Tryptic Soy Agar and incubated at 36± 1℃ for 48± 2 hours.

Preparation of Test Culture

- Test microorganisms are received from a reputable source (ATCC or equivalent) and revived and/or cultured according to the manufacturer's instructions.
- An aliquot of culture is supplemented with sterile glycerol to final concentration of 20%(v/v), mixed and placed in a -70± 10℃ freezer and represents the library stock culture.
- A loop-full of library stock culture is struck to Tryptic Soy Agar and incubated at the temperature appropriate for each test microorganism (see *Test microorganism Growth and Incubation Requirements).*
- A daily culture is initiated from the most recent monthly working stock culture for each microorganism and transferred at least once daily but no more than four prior to initiation of the culture. See *Test Microorganism Growth and Incubation Requirements.*
- The test culture is initiated by transferring a loop full of the last daily culture into each of an appropriate number of test tubes, each containing 10mL appropriate growth broth. See *Test Microorganism Growth and Incubation Requirements.*
- Test culture tube(s) are incubated for 48± 2 hours at the temperature appropriate for test system growth.
- Each test culture, with the exception of *P. aeruginosa*, is gently vortexed and allowed to stand at room temperature for ≥ 10 minutes.
- Prior to vortexing, *P. aeruginosa* test culture is carefully decanted into a separate sterile 50 mL conical tube making sure not to disturb and transfer the pellicle. The culture is then vortexed and allowed to stand at room temperature for ≥ 10 minutes.

Preparation of Test Culture Inoculum

- Enough volume of test culture is centrifuged and pellet is suspended and diluted in sterile PBS to yield a concentration of 1-5 x 10^7 CFU/ml.
- Dilute test cultures are supplemented with Triton X-100 to a final concentration of 0.05-0. 10% (v/v) and fetal bovine serum to a final concentration of 5% (v/v).
- Dilute test culture inocula are to be used within 4 hours of dilution.
- Each prepared and diluted test culture inoculum is serially diluted in sterile PBS and plated to the appropriate growth supporting agar (see *Test Microorganism Growth and Incubation Requirements)* to verify concentration (CFU/ml).
- Plates are incubated as detailed in the section incubation of plates and controls.

Preparation of Re-inoculation Culture Inoculum

- Each test culture is diluted in sterile PBS to yield a concentration of approximately ≥ 1 x 10^5 CFU/ml.
- Dilute re-inoculation cultures are supplemented with Triton X-100 to a final concentration of 0.1% (v/v) and fetal bovine serum to a final concentration of 5.0% (v/v).
- Dilute re-inoculation culture inocula are to be used within 8 hours of dilution, and for re-inoculation of treated and control carriers between each laundering cycle.
- Each prepared and diluted re-inoculation culture inoculum is serially diluted in sterile PBS and plated to the appropriate growth supporting agar (see *Test Microorganism Growth and Incubation Requirements)* to verify concentration (CFU/ml).
- Plates are incubated as detailed in the section incubation of plates and controls.

Treated and Control Carrier Inoculation and Exposure to Diluted Test Culture Inoculum

- Using a calibrated micropipette, a 0.10 mL volume of test culture is used to carefully inoculate, via spot inoculation, each test carrier, taking care to stay within 3mm of the edge of the test carrier. Petri dish lids are replaced after inoculation.
- A total of 6 replicates per test microorganism are inoculated for treated (laundered and unlaundered, 6 for contact time incubation and 3 for initial numbers control), and total of 6 control carriers per microorganism (3 for initial numbers control and 3 for contract time incubation).
- The Sponsor requested contact time starts immediately after inoculation and carries are left undisturbed at ambient temperature (23± 2℃) for the duration of the contact time.

Determination of Starting Microorganism Concentration (Initial Numbers Control)

- After the dry time, three Treated (laundered and unlaundered) and three Control Carriers for each microorganism are harvested by placement in 20 mL neutralization broth containing glass beads using flame-sterilized forceps.
- The neutralization medium tube is vortexed on maximum speed setting for 30 ± 5 seconds.
- Each neutralization tube used for elution of carriers is serially diluted 1:10, in 0.9 mL centrifuge tubes containing sterile PBS and plated to determine the initial concentration in CFU/carrier.

Determination of Final Carrier Viable Microorganism Concentration

- After the contact time has elapsed, three Control Carriers and three Treated Carriers (laundered and unlaundered) per test microorganism are harvested to 20 mL neutralization medium containing sterile glass beads using flame-sterilized forceps.
- Each neutralization medium tube is vortexed on maximum speed setting for 30± 5 seconds.

Each neutralization tube used for elution of carriers is serially diluted 1:10, using sterile PBS and plated to determine the final concentration in CFU/carrier.

Procedure for Continuous Bacterial Reduction

Test Microorganism Growth and Incubation Requirements

- *S. aureus,* and *P. aeruginosa* test and re-inoculation cultures are cultured in AOAC Nutrient Broth at 36± 1℃ for 24± 2 hours (daily culture) or 48± 2hours (test culture). All enumeration and controls are plated using Tryptic Soy Agar and incubated at 36± 1℃ for 48± 2 hours.

Preparation of Cultures Used in the Study

Note: The dilutions below may be modified to yield appropriate CFU/Counts for a particular test microorganism based on the final CFU/ml for a given culture (See Experimental Success Criteria below).

- An isolated colony is transferred from the most recent monthly working stock transfer to 10 ml AOAC Nutrient Broth and all test microorganisms and incubated at 35±2℃ for 24± 2 hours. A minimum of 1 daily culture is required to generate subsequent cultures (see below) by transferring a loopful of the 24 hour culture to sterile Synthetic or Nutrient Broth. Daily transfers are not to exceed a transfer number of 4(t=4) and prior to inoculating the Initial Inoculation Culture, Re-inoculation Culture or Final Test culture.
- The Initial Inoculation Culture (transfer≤ 5) is incubated for 48-54 hours at 35±2℃. The culture is vortexed for 3-4 seconds and allowed to sit for ≥ 10 minutes, except for *P. aeruginosa* where the culture is decanted and collected in a sterile 50 ml conical tube (or equivalent), thus leaving behind the pellicle prior to vortexing. A1:4 dilution is performed in sterile RO water (e.g. 2.5 ml culture plus 7.5 ml RO water) and an aliquot of the final 1:4 dilution supplemented with fetal bovine serum to yield a 5% (v/v) final FBS concentration. Alternatively, the RO water diluent may be supplemented with the appropriate volume of FBS to yield a 5% (v/v) suspension upon dilution of the culture to minimize preparation steps. The final FBS-supplemented suspension is vortexed 3-4 seconds to mix.
- The Re-inoculation Culture (transfer ≤5) is incubated for 48-54 hours at 35± 2℃. The culture is vortexed for 3-4 seconds and allowed to sit for ≥ 10minutes, except for *P. aeruginosa* where the culture is decanted and collected in a sterile 50 ml conical tube (or equivalent), thus leaving behind the pellicle prior to vortexing. A 1:400 dilution is performed in sterile RO water (e.g. 0.025 ml of culture in 10 ml RO water) and an aliquot of the final 1:400 dilution supplemented with fetal bovine serum to yield a 5% (v/v) final FBS concentration. Alternatively, the RO water diluent may be supplemented with the appropriate volume of FBS to yield a 5% (v/v) suspension upon dilution of the culture to minimize preparation steps. The final FBS-supplemented suspension is vortexed for 3-4 seconds to mix.

Treated and Control Carrier Inoculation with "Initial Inoculation Culture"

- Using a calibrated micropipette, a 0.100 mL volume of test culture is used to carefully inoculate, via spot inoculation, each carrier, taking care to stay within 3mm of the edge of the test carrier.

- A total of 3 replicates per test microorganism are inoculated for treated laundered carriers per microorganism
- Inoculated carriers are allowed to dry for 10-20 minutes at ambient temperature (23± 2℃).

Determination of Starting Microorganism Concentration (Initial Numbers Control)

- After the dry time, three Control Carriers for each microorganism are harvested by placement in 20 mL neutralization broth containing glass beads using flame-sterilized forceps.
- The neutralization tube is vortexed on maximum speed setting for 30± 5 seconds.
- Each neutralization tube used for elution of carriers is serially diluted 1:10, 0.9 mL centrifuge tubes containing sterile PBS and plated to determine the initial concentration in CFU/carrier.

Re-inoculation Carrier Control

- Two sterile carriers are inoculated upon initial use of each prepared Re-inoculation Culture and dried alongside treated and control carriers.
- After the dry time, three control carriers for each microorganism are harvested by placement in 20 mL neutralization broth containing glass beads using fame-sterilized forceps.
- The neutralization tube is vortexed on maximum speed setting for 30± 5 seconds.
- Each neutralization tube used for elution of carriers is serially diluted 1:10, in 0.9 mL centrifuge tubes containing sterile PBS and plated to determine the initial concentration in CFU/carrier.

Hours*	**Minimum CFU/Carrier**	**Inoculation/Re-inoculation Procedure**	**Log_{10} Reduction**
0-1	$1x10^6$ for test Bacteria	Inoculation of All Carriers with initial inoculation culture	None
1-24	$1x10^4$ for test Bacteria	Re-inoculation (1)	None
		Re-inoculation (2)	
		Re-inoculation (3)	
		Re-inoculation (4)	
		Re-inoculation (5)	
		Re-inoculation (6)	
		Re-inoculation (7)	
		Re-inoculation (8)	
		Re-inoculation (9)	
		Re-inoculation (10)	
		Re-inoculation (11)	
24	$1x10^8$ for test Bacteria	Residual Self-Disinfection Test after 10 minute contact time	5 log_{10} reduction

Inoculum Concentration Determinations

- The concentrations (CFU/ml) of the Initial Inoculation Culture, Re-inoculation Culture, and final Test Culture are determined by serial dilution in sterile PBS and plating in duplicate to TSA.

Continuous Reduction Efficacy Determination

- Continuous reduction efficacy is determined for all carriers (Test and Control) after all re-inoculations are complete, and at least 24 hours after initial inoculation but not to exceed 48 hours.
- Using a calibrated micropipette, a 0.100mL volume of test culture is used carefully inoculate, via spot inoculation, each carrier, taking care to stay within 3mm of the edge of the test carrier.
- A total of 3 replicates per test microorganism are inoculated for treated laundered carriers, 3 laundered control carriers per test microorganism.
- The Sponsor requested contact time starts immediately after inoculation and carriers are left undisturbed at ambient temperature (23$\pm$ 2℃) for the duration of the contact time.
- After the contact time, carriers are harvested by placement in 20 mL neutralization broth containing glass beads using flame-sterilized forceps.
- The neutralization tube is vortexed on maximum speed setting for 30$\pm$ 5 seconds.
- Each neutralization tube used for elution of carriers is serially diluted 1:10, in 0.9 mL centrifuge tubes containing sterile PBS and plated to determine viable CFU/carrier on treated, and control carriers.

Controls

Neutralization Control

- At least 3 replicates are performed per test microorganism.
- 3 unlaundered and laundered Test Carriers from each lot tested are harvested to neutralization medium in a manner equivalent to that performed in the test as described above, and then inoculated with 1.0 mL of dilute test microorganism that results in a final suspension that contains $\leq$ 100 CFU/mL of test microorganism.
- 3 neutralization medium tubes are inoculated directly with the same inoculum described above.
- All neutralization tubes in this step are enumerated using standard dilution and plating.
- Neutralization samples are held at ambient temperature for $\geq$ 10 minutes.
- Geometrically overage counts from test, control, and direct inoculum tubes must be within 0.5 $\log_{10}$ of one another to demonstrate neutralization of the active ingredient by the neutralization medium chosen for the test.

Media sterility Control(s)

- A 1 mL aliquot PBS and neutralizing broth media are plated to determine media sterility. One plate containing only the growth medium used in this study is incubated to determine media sterility.

"Soil" Sterility Control (Fetal Bovine serum)

- 0.100ml of "soil" is plated to appropriate agar for sterility confirmation.

Media Growth and Culture Purity Control

- A loop full of each test microorganism test culture is struck to the appropriate growth agar to achieve isolated colonies in order to confirm culture purity as well as serve as the media growth control.

Sterility Control (Treated and Control Carriers)

- A subset of control and treated carriers (laundered and unlaundered) representing a minimum of 10% of carriers to be tested on the day of efficacy testing, are harvested and neutralized as detailed in *Determination of Final Carrier Viable Microorganism Concentration* and the 20ml volume passed through a 0.45µm filter. The filter rinsed with 40± 5ml sterile PBS and the filter placed directly on the appropriate growth agar plate.
- The plates containing the filters are incubated as detailed in the section incubation of plates and controls.

Incubation of Plates and Controls

- All enumeration and control plates are incubated for 48± 4 hours at 36± 1℃ and for all test microorganisms.

Success Criteria

- The experimental success (controls) criteria follow for Initial Reduction (Non-Continuous Claim):
  1. All media sterility controls must be negative for growth.
  2. Carrier contamination control must demonstrate negligible contamination.
  3. The media growth control must be positive for growth.
  4. All test microorganisms must demonstrate culture purity.
  5. Neutralization is validated as described previously.
  6. Soil sterility control is negative for growth.
  7. Re-inoculation Culture enumerations demonstrate $\geq 1 \times 10^4$ CFU/carrier.
  8. Initial Numbers Control enumeration demonstrate $\geq 1 \times 10^6$ CFU/carrier.
  9. Final (post contact time) Control Carrier count enumeration results demonstrate $\geq 1 \times 10^8$ CFU/carrier.

- The experimental success (controls) criteria follow for Continuous Reduction:
  1. All media sterility controls must be negative growth.
  2. Carrier contamination control must demonstrate negligible contamination.
  3. The media growth control must be positive for growth.
  4. All test microorganisms must demonstrate culture purity.
  5. Neutralization is validated as described previously.
  6. Soil sterility control is negative for growth.

7. Initial inoculation control carriers must demonstrate an average $\geq 1 \times 10^6$ CFU/carrier for a valid test.
8. Re-inoculation control carriers must demonstrate an average $\geq 1 \times 10^4$ CFU/carrier for a valid test.
9. Final efficacy control carriers must demonstrate an average $\geq 1 \times 10^8$ CFU/carrier for a valid test.

Test substance performance criteria

The results must show a bacterial reduction of at least 99.999% for treated carriers (laundered and unlaundered) and when compared to the parallel untreated control.

Reporting

- The report will include, but is not limited to, identification of the sample, date received, dates on which the test was initiated and completed, identification of the bacterial strains used, description of media and reagents, description of the methods employed, tabulated results and conclusion as it relates to the purpose of the test, and all other items required by 40 CFR Part 160.185. A draft final report will be provided to the Sponsor for review prior to finalization.
    - The study report and corresponding data will be held in the archives of Biodetek Laboratory for at least 2 years after the date of final report. After 2 years documents may be returned to the study sponsor for archiving
    - Archiving test substances is the responsibility of the Sponsor

Quality Control

- The study will be conducted in accordance with the Performing Laboratories Quality Management System and will undergo a full quality assurance review. All protocol amendments will be fully recorded and reported as well as any deviation(s) from the protocol

References

American Association of Textile Chemists and Colorists. AATCC 100. Antimicrobial Finishes on Textiles: Assessment of. (2012). AATCC 100-2012